Arch. Pharm. Res. Vol.21, No.2, pp. 212-216, 1998

Coupled Column Chromatography in Chiral Separation of Salmeterol

Kyeong Ho Kim[1], Hyeong Won Yun[1], Hyun Ju Kim[1], Hyun Ji Park[2] and Pok Wha Choi[1]

[1]College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea and [2]Department of Pharmacy, Seoul National University Hospital, Seoul 110-744, Korea

(Received December 12, 1997)

A coupled achiral-chiral high-performance liquid chromatographic system has been developed for the determination of the enantiomers of salmeterol, S-(+)-salmeterol and R-(-)-salmeterol in urine. The salmeterol was separated from the interfering components in urine and quantified on the silica column, and the enantiomeric composition was determined on a Sumichiral OA-4700 chiral stationary phase. The two columns were connected by a switching valve equipped with a silica precolumn. The precolumn was used to concentrate the salmeterol in the eluent from the achiral column before backflushing onto the chiral phase. The coupled system was validated.

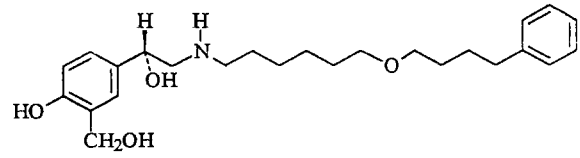
Key words : Chiral separation, HPLC, Coupled column chromatography, Salmeterol, Enantiomer, Column switching

INTRODUCTION

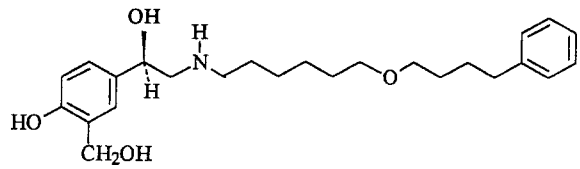
Salmeterol xinafoate is a long-acting β_2-adrenoreceptor agonist, used in the treatment of asthma, that has bronchodilator and anti-inflammatory action. It is marketed, like many other chiral drugs, as a racemate (Broden *et al.*, 1991; Johnson, 1990, 1991 & 1993). However, it is well established, that single enantiomers are often much more potent and have reduced side effects compared to their racemates (Ariëns *et al.*, 1992). For many other β_2-agonist, the active isomer is the R-(-)-salmeterol (Fig. 1) which is 40 times more potent than S-(+)-salmeterol (Johnson, 1995). In order to develop a drug which contains only R-(-)-salmeterol, a sensitive, accurate and precise method for the determination of each enantiomer in urine samples for safety evaluation and pharmacokinetic studies.

The enantiomers of salmeterol can be directly resolved on a Sumichiral OA-4700 chiral stationary phase using *n*-hexane-1,2-dichloroethane-methanol-trifluoroacetic acid (240:140:25:1, v/v) as a mobile phase with a resolution of 1.24. However, this separation could not be dirctly applied to urine samples due to interference from endogenous compounds. To overcome this problem, a coupled achiral-chiral high-performance liquid chromatographic system (Chu *et al.*, 1989 & 1992; Huber *et al.*, 1973; Oda *et al.*, 1991; Rizzi, 1990 & 1991; Wainer *et al.*, 1988) was developed. In this system, the salmeterol was separated from interferences in biological matrix and quantitated on an achiral silica column. The eluent containing salmeterol was then transferred to a silica precolumn where the salmeterol was concentrated. The target compound was then backflushed onto the Sumichiral OA-4700 chiral column where R-(-)-salmeterol and S-(+)-salmeterol were stereochemically resolved and the enantiomeric ratio was determined (Fig. 2).

This paper reports the development and validation of this achiral-chiral coupled system of the analysis of



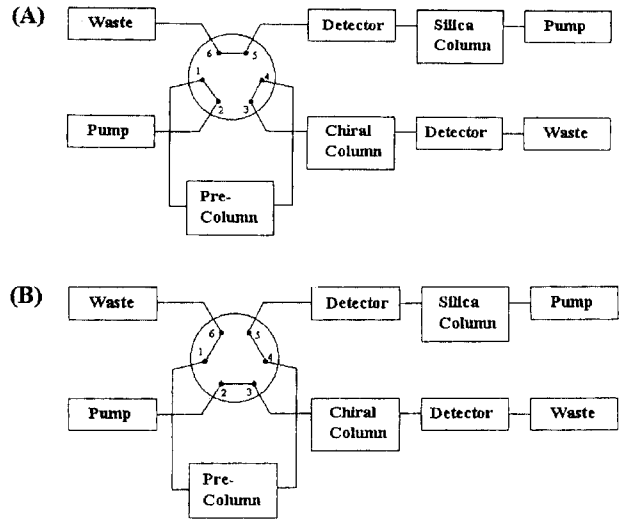
S-(+)-Salmeterol



R-(-)-Salmeterol

Fig. 1. Structures of enantiomers of salmeterol.

Correspondence to: Kyeong Ho Kim, College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea



Position	Time (min)	Function
A	0.00~23.49	Waste
B	23.50~25.50	Load salmeterol on silica precolumn
A	25.51~45.00	Elute salmeterol from chiral column

Fig. 2. Schematic diagram of the coupled achiral-chiral column chromatographic system and positions and functions of switching.

salmeterol enantiomers in urine samples.

MATERIALS AND METHODS

Materials and equipment

Racemic salmeterol xinafoate was a gift from Glaxo Korea (Ansan, Korea). The internal standard, bamethane hydrochloride was purchased from Sigma (St. Louis, MO, USA). n-Hexane, 1,2-dichloroethane, ethylacetate and methanol were purchased from J.T Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid was from Aldrich (Milwaukee, WI, USA). R-(-)-Salmeterol ans S-(+)-salmeterol were prepared from racemic salmeterol by semi-preparative chiral high-performance liquid chromatography. All other chemicals were reagent grade and used as purchased.

The achiral chromatography was performed with a Shimadzu (Kyoto, Japan) liquid chromatography system composed of SCL-6B system controller, LC-9A pump, Rheodyne 7725i injector, RF535 fluorescence detector set at 280nm for excitation and 305nm for emission, C-R6A integrator and GL Si-100 HPLC column (5 μm, 4.6×250 mm, GL Sciences Inc., Tokyo, Japan).

The separation of salmeterol racemate and the internal standard bamethane from the urine components was accomplished on the silica column using a mobile phase composed of *n*-hexane-1,2-dichloroethane-ethylacetate-methanol-trifluoroacetic acid (240:210:150:25:1, v/v). The analysis were carried out using a flow rate of 1.0 ml/min and ambient temperature.

The chiral chromatography was performed with Shimadzu LC-9A pump, TSP FL-3000 fluorescence detector (Thermo Separation Products Inc., FL, USA) and Shimadzu C-R4AD data processor. The chiral column was Sumichiral OA-4700 (5 μm, 4.6×250 mm, Sumika Chemical, Osaka, Japan). The stereochemical separation of R-(-)-Salmeterol and S-(+)-Salmeterol was accomplished using a mobile phase n-hexane-1,2-dichloroethane-ethylacetate-methanol-trifluoroacetic acid (240:210:150:45:1, v/v). The analysis was carried out using a flow rate 1.0 ml/min and ambient temperature. The two systems were connected through Shimadzu FCV-2AH high-pressure flow-channel selection valve equipped with a precolumn (4.6×30 mm) which was packed with 5 μm silica gel.

Sample preparation

One ml aliquot of urine sample and 100 μl of internal standard solution of bamethane (0.1 μg/ml) were transferred to a prelabelled culture tube and mixed. The solid-phase (silica) extraction column was preconditioned by washing with 10 ml of methanol followed by 10 ml of n-hexane. The washing solvents was allowed to pass through with minimum vacuum (<50 mmHg) which was released immediately after the solvents eluted from all the cartridges. The previously mixed urine sample was transfered into the preconditioned column and minimum vacuum was applied. When the urine sample in the cartridge reservoir had been removed, the vacuum was increased to 500 mmHg for 2 min and then released. Each column was then washed with 2 ml of n-hexane followed by 1 ml of acetonitrile under minimum vacuum until all the washing solvents eluted from the cartridges. The column was dried by full vacuum for an additional 5 min. Salmeterol and bamethane were then eluted from the silica adsorbent by rinsing with 5 ml of isopropanol under minimum vacuum until no effluent was observed. The effluent was evaporated to dryness under the nitrogen gas at room temperature and the residue was reconstituted in 200 μl of achiral mobile phase and 100 μl was injected into the achiral HPLC system.

Standard curves and recovery

A standard curve for total salmeterol [R-(-)-salmeterol and S-(+)-salmeterol] urine concentrations was prepared by addition of 20, 50, 100, 250, 500 ng/ml to drug-free urine. The standard curve was constructed by plotting the salmeterol/bamethane peak area ratios versus known salmeterol concentrations.

The percent recovery and reproducibility of this method were investigated using drug-free urine samples spiked with 50, 100, 500 ng/ml racemic salmeterol with n=5 at each level.

RESULTS AND DISCUSSION

Chiral semi-preparative HPLC of R-(-)-salmeterol and S-(+)-salmeterol and determination of the elution order

Salmeterol xinafoate was dissolved in 10 ml of mobile phase. This solution was injected into the semi-preparative chiral HPLC system and resolved into each enantiomer on the Sumichiral OA-4700 chiral column (5 μm, 8.0×250 mm) by the n-hexane-1,2-dichloroethane-methanol-trifluoroacetic acid (240:140:20:1, v/v) as a mobile phase at room temparature and flow rate of 4 ml/min monitered at 280 nm UV.

Fractions containing single enantiomers were collected and evaporated to dryness under nitrogen stream. Optical purity was determined by the chiral HPLC using Sumichiral OA 4700 analytical column (5 μm, 4.6 ×250 mm) and n-hexane-1,2-dichloroethane-ethylacetate-methanol-trifluoroacetic acid (240:210:150:40:1, v/v) as a mobile phase. Optical purity of each enantiomer was not less than 99.9%. The direction of rotation (+/-) was determined using a Jasco DIP-1000 digital polarimeter. S-(+)-salmeterol (Hett *et al.*, 1994) was eluted first (Fig. 3). The retention time of each enantiomer was 26.83 and 31.44 min, respectively.

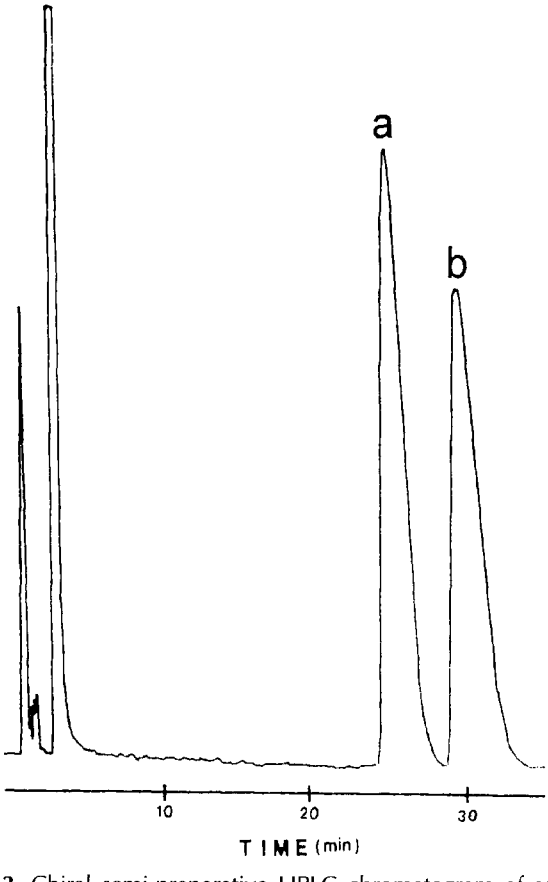


Fig. 3. Chiral semi-preparative HPLC chromatogram of salmeterol enantiomers. Column; Sumichiral OA-4700 (5 μm, 8.0× 250 mm), mobile phase; *n*-hexane : 1,2-dichloroethane : methanol : trifluoroacetic acid (240 : 140 : 20 : 1, v/v), detector; UV 280 nm. Peak a; S-(+)-salmeterol, peak b; R-(-)-salmeterol.

Optimization of coupled achiral-chiral chromatographic system

The ethylacetate content in the achiral mobile phase had a profound effect on the separation factors of the salmeterol, bamethane and three endogeneous peaks, as shown in Fig. 4. As the content of ethylacetate increased, the capacity factors of salmeterol and bamethane decreased but the capacity factors of three unidentified intefering substances did not changed. In the chiral chromatographic system, the capacity fac-

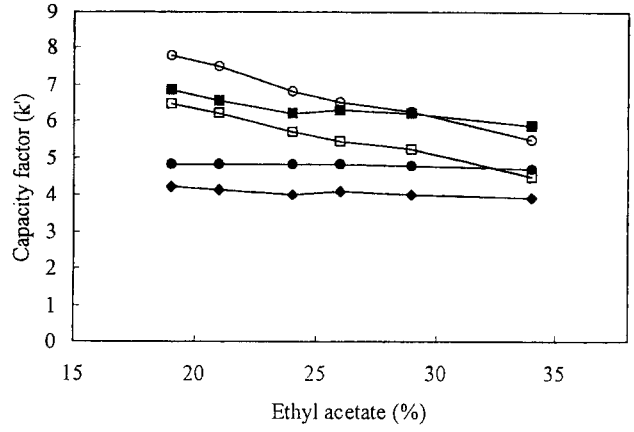


Fig. 4. The effect of ethyl acetate concentration in the achiral mobile phase on the capacity factors of salmeterol, bamethane (internal standard) and interference peaks in the achiral HPLC system. Mobile phase; *n*-hexane:1,2dichloroethane: methanol:trifluoroacetic acid (240:210:25:1, v/v) with varying content of ethyl acetate. ○: Salmeterol racemate, □: Bamethane, ■: Interference peak1, ●: Interference peak2, ◆: Interference peak3.

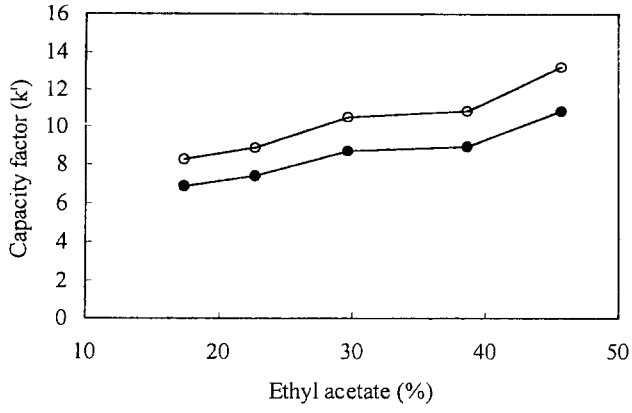


Fig. 5. The effect of ethyl acetate concentration in the chiral mobile phase on the capacity factors of salmeterol enantiomers in the chiral HPLC system. Mobile phase; *n*-hexane: 1,2-dichloroethane:methanol:Trifluoroacetic acid (240:210 :25:1, v/v) with varying content of ethyl acetate. ○: R-(-)-Salmeterol, ●: S-(+)-Salmeterol.

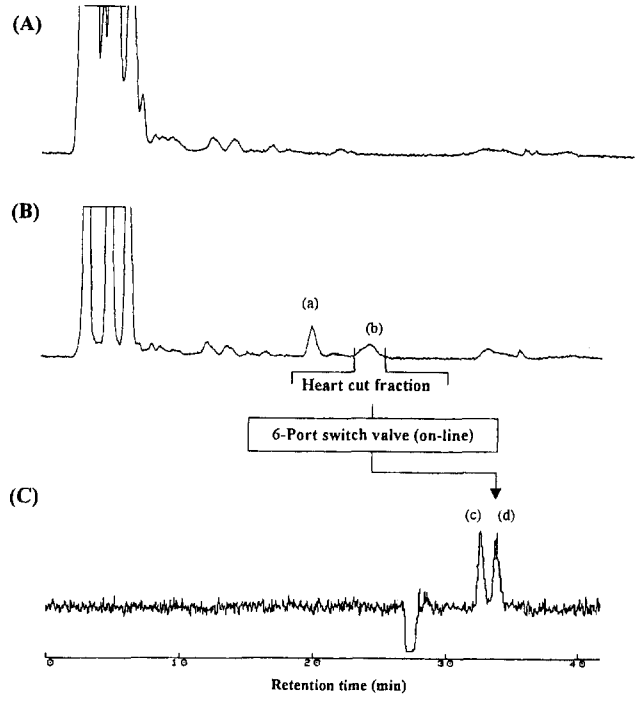


Fig. 6. Representative chromatograms of urine samples on the coupled achiral-chiral chromatographic system. (A) achiral chromatogram of urine blank (B) achiral chromatogram of urine spiked with 100 ng/ml salmeterol (C) chiral chromatogram after column switching. Peak a; Bamethane, peak b; salmeterol racemate, peak c; S-(+)-salmeterol, peak d; R-(-)-salmeterol. Achiral column; Inertsil SIL 100-5 (5 μm, 4.6×250 mm), detector; Ex280 nm Em305 nm, mobile phase; *n*-hexane : ethyl acetate : 1,2-dichloroethane : methanol : trifluoroacetic acid (240 : 150 : 210 : 25 : 1, v/v). Chiral column; Sumichiral OA-4700 (5 μm, 4.0×250 mm), detector; Ex280 nm Em 305 nm, mobile phase; *n*-hexane : ethyl acetate : 1,2-dichloroethane : methanol : trifluoroacetic acid (240 : 150 : 210 : 45 : 1, v/v).

tor of each enantiomer of salmeterol increased and bandbroadening effect was found (Fig. 5).

Typical chromatograms of the coupled achiral-chiral chromatographic system for the chiral separation of salmeterol enatiomers are presented in Fig. 6. Under the described achiral chromatographic conditions, salmeterol and the internal standard produced symmetric peaks which occured at 19.87 and 24.27 min. Three endogeneous peaks occurring at 14.04, 16.97 and 22.18 min, respectively, were clearly separated from salmeterol and its internal standard (Fig. 6B). Under the described chiral chromatographic condition, the retention times of S-(+)-salmeterol and R-(-)-salmeterol were 32.16 and 33.84 min, respectively; the observed stereoselectivity factor (α) was 1.04 and stereochemical resolution (Rs) was 1.56.

Recovery and precision

The standard curve for the salmeterol was linear over the range investigated and the equation describing the curve was y=0.0102×-0.0076 with a correlation coefficient of 0.9998. The recovery test was carried out with urine samples spiked with 50, 100 and 500 ng/ml racemic salmeterol. The results are represented in Table I. The extraction efficiency averaged 102.3%.

Table I. Recovery test for the R-(-)-salmeterol and S-(+)-salmeterol in human urine (n=5)

Amount added (ng/ml)	Component	Amount recovered (ng/ml)	Recovery (%)	Precision (C.V.)
25	R-(-)-salmeterol	25.79	103.16	7.06
	S-(+)-salmeterol	26.92	107.68	7.32
50	R-(-)-salmeterol	51.15	102.29	7.65
	S-(+)-salmeterol	51.99	103.99	7.04
250	R-(-)-salmeterol	246.06	98.42	0.75
	S-(+)-salmeterol	246.52	98.61	0.75

The S-(+)-salmeterol/R-(-)-salmeterol peak area ratio of racemic salmeterol was investigated over the urine concentration range 0.05~2.0 μg/ml. The peak area ratio was 1.00 over this range.

The total salmeterol concentrations ([Salmeterol]) were determined on the achiral section of the coupled chromatographic system and the percentage of S-(+)-Salmeterol [%S-(+)-Salmeterol] and R-(-)-salmeterol [%R-(-)-Salmeterol] were determined on the chiral section. The both determinations were carried out in a single experiment. The total amounts of each enantiomers were calculated using the following equations:

total S-(+)-salmeterol=[Salmeterol]× [% S-(+)-Salmeterol]
total R-(-)-salmeterol=[Salmeterol]× [% R-(-)-Salmeterol]

The precisions of the assay for the S-(+)-Salmeterol and R-(-)-Salmeterol were averaged 5.15% and 5.24%, respectively.

In conclusion, one of the major problems encountered in the application on HPLC chiral stationary phase to the analysis of biological samples is the coelution of the enantiomers and interfering compounds from the matrix because of the low sample capacity. One solution to this problem is the use of achiral-chiral coupled column systems in which the enantiomers are separated from interferences from the biological matrix on the achiral phase and then switched to the chiral stationary phase for enantiometric analysis. The coupled achiral-chiral system used for the determination of salmeterol enantiomers in urine has only a single injector and used a precolumn instead of a loop, which increased the efficiency of the transfer between achiral and chiral systems. The assay described in this paper can be used for the pharmacokinetic study of salmeterol enantiomer.

REFERENCES CITED

Ariëns, E. J., Racemic Therapeutics: A source of prob-

lems to chemists and physicians. *Anal. Proc.*, 29, 232-234 (1992).

Broden, R. N. and Faulds, D., Salmeterol xinafoate. A review of its pharmacological properties and therapeutic potential in reversible obstructive airways disease. *Drugs,* 42, 895-912 (1991).

Chu, K., Shieh, S., Wu, S., Hu, O. Y., Enantiomeric separation of a cardiotonic agent pinobendan and its major active metabolite, UD-CG 212 BS, by a coupled achiral-chiral normal-phase high performance liquid chromatography. *J. Chromatogr. Sci.*, 30, 1-6 (1992).

Chu, Y. and Wainer, I. W., Determination of the enantiomers of verapamil and norverapamil in serum using coupled achiral-chiral high-performance liquid chromatography. *J. Chromatogr.,* 497, 191-200 (1989).

Hett, R., Stare, R. and Helquist, P., Enantioselective synthesis of salmeterol via asymmetric borone reduction. *Tetrahedron Letters,* 35, 9375-9378 (1994).

Huber, J. F. K., Van der Linden, R., Ecker, E. and Oreans, M., Column switching in high-pressure liquid chromatography. *J. Chromatogr.,* 83, 267-277 (1973).

Johnson, M., The pharmacology of salmeterol. *Lung,* suppl: 115-119 (1990).

Johnson, M., The preclinical pharmacology of salmeterol: non-bronchodilator effects. *Eur. Respir. Rev.*, 1(4), 257-260 (1991).

Johnson, M., Salmeterol: a novel drug for the treatment of asthma. *Agents Action,* 34(Suppl.), 79-95 (1991).

Johnson, M., β_2-Antagonists as anti-inflammatory therapeutics in the lung. *Inflam. Diseas. Ther.*, 27-45 (1993).

Johnson, M., Salmeterol. *Med. Res. Rev.*, 15, 225-257 (1995).

Oda, Y., Asakawa, N., Kajima, T., Yoshida, Y. and Sato, T., Column-switching high-performance liquid chromatography for on-line simultaneous determination and resolution of enantiomers of verapamil and its metabolites in plasma. *Pharm. Res.*, 8, 997-1001 (1991).

Rizzi, A. M., Coupled column chromatography in chiral separations I. Enantiomeric separation on swollen microcrystalline cellulose triacetate columns after a preseparation on a nonchiral alkylsilica column. *J. Chromatogr.,* 513, 195-207 (1990).

Rizzi, A. M. and Plank, C., Coupled column chromatography in chiral separations: systems employing β-cyclodextrin phases for chiral separation. *J. Chromatogr.,* 557, 199-213 (1991).

Wainer, I. W. and Stiffin, R. M., Direct resolution of the stereoisomers of leucovorin and 5-methyltetrahydrofolate using a bovine serum albumin high-performance liquid chromatographic chiral stationary phase coupled to an achiral phenyl column. *J. Chromatogr.*, 424, 158-162 (1988).